Intracellular localization of a group II chaperonin indicates a membrane-related function

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Chaperonins are protein complexes that are believed to function as part of a protein folding system in the cytoplasm of the cell. We observed, however, that the group II chaperonins known as rosettasomes in the hyperthermophilic archaeon *Sulfolobus shibatae*, are not cytoplasmic but membrane associated. This association was observed in cultures grown at 60°C and 76°C or heat-shocked at 85°C by using immunofluorescence microscopy and in thick sections of rapidly frozen cells grown at 76°C by using immunogold electron microscopy. We observed that increased abundance of rosettasomes after heat shock correlated with decreased membrane permeability at lethal temperature (92°C). This change in permeability was not seen in cells heat-shocked in the presence of the amino acid analogue azetidine 2-carboxylic acid, indicating functional protein synthesis influences permeability. Azetidine experiments also indicated that observed heat-induced changes in lipid composition in *S. shibatae* could not account for changes in membrane permeability. Rosettasomes purified from cultures grown at 60°C and 76°C or heat-shocked at 85°C bind to liposomes made from either the bipolar tetraether lipids of *Sulfolobus* or a variety of artificial lipid mixtures. The presence of rosettasomes did not significantly change the transition temperature of liposomes, as indicated by differential scanning calorimetry, or the proton permeability of liposomes, as indicated by pyranine fluorescence. We propose that these group II chaperonins function as a structural element in the natural membrane based on their intracellular location, the correlation between their functional abundance and membrane permeability, and their potential distribution on the membrane surface.

Nearly all organisms respond to heat and other stresses by synthesizing a small subset of proteins known as heat shock, or stress, proteins (HSPs), the production of which correlates with an increased tolerance for lethal conditions (1, 2). There is compelling evidence that HSPs participate in this so-called acquired tolerance (3–5), although which HSPs are critical and how they function remains a topic of active research and seems to differ for different organisms or cell types (6). In the hyperthermophilic archaeon *Sulfolobus shibatae*, which grows optimally at 83°C (7), acquired thermotolerance at lethal temperatures (>90°C) correlates with the increased synthesis of primarily two 60-kDa HSPs known as TF55 α and β (8–10). These proteins are isolated from cells as subunits of double-ring complexes called rosettasomes that reportedly share structural and functional features with chaperonins (11).

Sequence comparisons of chaperonin subunits and structural analyses of the double rings have led to the recognition of two groups of chaperonins (12). The so-called group I chaperonins found in bacteria and the chloroplasts and mitochondria of Eukarya are composed of identical or two closely related subunits arranged in two stacked rings with seven subunits each. The group II chaperonins found in Archaea and Eukarya are composed of identical or diverse subunits arranged in rings of eight or nine subunits. Chaperonins in both groups are reported to play a role in refolding stress-damaged proteins or folding newly synthesized proteins *in vivo* (for review see ref. 13).

The hypothesis that protein folding is the primary function of all chaperonins stems from observations that they are all composed of 60-kDa HSPs (HSP60s), heat and other HSP-inducing stresses are known to unfold proteins (14), and for group I chaperonins there is evidence for folding or refolding proteins (13). For archaeal group II chaperonins, although it has been demonstrated that they are able to recognize and bind to unfolded proteins (11) and in some cases promote their refolding *in vitro* (15, 16), there is growing evidence they may have other functions *in vivo* (17–20).

To explore the role of group II chaperonins *in vivo*, we investigated the intracellular location of rosettasomes in *S. shibatae* under normal, heat, and cold shock conditions. We reasoned that if rosettasomes are involved in protein folding they should be located in the cytoplasm where most protein folding is presumed to occur. We observed, however, that rosettasomes are membrane associated under all conditions. We therefore investigated whether changes in their abundance correlate with changes in membrane permeability, whether they bind to lipid components of the *S. shibatae* or model membranes, and whether their presence on liposomes influences the structure of the lipids or their permeability to protons. Based on our observations, we hypothesize that these group II chaperonins function as a membrane "skeleton" in *S. shibatae* that interacts with lipids and/or membrane-associated proteins to impact the permeability and stability of the cytoplasmic membrane.

Materials and Methods

Cell Culturing Conditions and Rosettasome Purification. *S. shibatae* (German Collection Microorganisms and Cell Cultures strain 5389) was grown in liquid medium containing 0.2% sucrose as described (8). The medium was adjusted to pH 2.3 in all cultures except for those used in propidium iodide experiments, which were adjusted to pH 3.2. All cultures were grown in shaking water baths (Boekel Grant, Feasterville, PA, model ORS200) at 76°C and used in log-phase growth ($\approx 3 \times 10^8$ cells per ml). Temperature shifts were done by using equilibrated water baths.

To obtain 60°C rosettasomes, *S. shibatae* cultivated at 76°C overnight was transferred to 60°C and harvested by centrifugation when the cell density reached 2×10^9 cells per ml ($\approx$10 days). For 76°C rosettasomes, *S. shibatae* was cultivated at 76°C for $\approx$4 days. For 86°C rosettasomes, *S. shibatae* cultivated at 76°C was transferred to 86°C for 20 h. Rosettasomes were purified by previously described methods (20) with the following modifications: (*i*) buffer A included 10 mM $MgCl_2$ and 10 mM KCl to stabilize rosettasomes, (*ii*) the heating step to remove *Escherichia coli* proteins was omitted, and (*iii*) rosettasome-containing fractions from the DEAE column were concentrated and double rings were separated from subunits by using a 10–30% linear glycerol gradient centrifuged at 16,000 rpm for 17 h at 4°C in an

Abbreviations: HSP, heat shock (stress) protein; IFM, immunofluorescence microscopy.

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SW28 rotor (Beckman) before further purification by chromatography (Mono-Q, Pharmacia).

Microscopy. Immunofluorescence microscopy (IFM) was done essentially as described (21) with the following modifications. Microscopy grade fixative to final concentration 2.7% paraformaldehyde (Sigma) and 0.0083% glutaraldehyde (Sigma) were added to 250 μl of culture. The pH 2–3 of the culture medium prevented fixation of the S-layer proteins, which causes cell shrinkage, but the neutral pH of the cytoplasm allowed fixation inside cells. Fixed cells were attached to poly-L-lysine-coated multiwell glass slides and alkali-treated [10% 1 M Trizma base plus 90% *S. shibatae* sucrose growth medium (8)]. This stopped fixation and permeabilized cells. Immunolabeling was done for 1 h by using protein-A purified polyclonal antiserum at 38 μg/ml followed by FITC-conjugated secondary antibody (Jackson ImmunoResearch).

For membrane leakage experiments, propidium iodide (Molecular Probes) was added to a final concentration of 30 μM immediately before observing samples. Cells were transferred to glass slides and photographed by using differential interference contrast and fluorescence microscopy. Intact and permeable cells were quantified in three regions of a slide away from the edges of the cover glass, where we observed disproportionate cell damage presumably caused by evaporation. A mean and standard deviation were calculated.

Immuno-electron microscopy was done on high-pressure frozen samples (Balzers HPM 010). For freeze-substitution samples were kept at −90°C for 3 days in 2.0% OsO_4, 0.1% uranyl acetate, warmed to −20°C for ≈12 h and then warmed to 0°C for 1 h before rinsing three times in 100% acetone at room temperature (20 min total). Samples were infiltrated with Epon (EM Science) 1:1 resin/acetone (30 min), 2:1 resin/acetone (30 min), and pure resin and accelerator (60 min and overnight). A final change of resin with accelerator was polymerized in an oven (Ted Pella, Redding, CA) as described by the manufacturer. Sections (0.25 μm or 1 μm thick) were cut (Reichert Ultracut Microtome) and etched by using an Epoxy Removal kit (Polysciences) before immunolabeling as described (22). Polyclonal antibodies against *S. shibatae* rosettasome proteins and gold-conjugated secondary antibodies (Ted Pella) were used for labeling. Labeled sections were viewed in a Philips (Portland, OR) 410 transmission electron microscope.

Lipid Analyses and Liposome Preparation. Total lipids were extracted from lyophilized *S. shibatae* cells by a single-phase Bligh and Dyer procedure modified by the addition of 5% trichloroacetic acid to the aqueous phase (23, 24). The ether-bound isoprenoid moieties of the lipids were released by the addition of BBr_3 as follows. While flushing with nitrogen, 40 μl of 1.0 M BBr_3 in methylene chloride (Aldrich) was added to 50 μl of lipid extract in methylene chloride, and tubes were heated at 60°C for 2 h. After cooling to room temperature, samples were quenched with 1 ml of water and extracted four times with 1 ml of methylene chloride. The isoprenyl bromides were dried and reduced by the addition of 500 μl of superhydride solution (1.0 M lithium triethylborohydride in tetrahydrofuran, Aldrich) incubated under nitrogen at 70°C for 2 h. The reaction was quenched with 1 ml of water and extracted four times with hexane-chloroform (4:1). The primary products, biphytanes with 0, 1, 2, or 3 cyclopentane rings in the C40 chains were quantified by using a Hewlett–Packard 6890 GC equipped with a flame ionization detector and HP-5 (30 m by 0.25 mm, 0.25-μm film) column.

Liposomes were made from bipolar tetraether lipids generously provided by Parkson L.-G. Chong (Temple University, Phiadelphia) extracted from *Sulfolobus* as described (25). Liposomes were also made from model lipids obtained from Avanti Polar Lipids or Sigma. Stock lipid solutions in chloroform were dried under nitrogen and then under vacuum. To make liposomes, a lipid suspension (10 mg/ml) was freeze-thawed three times in an appropriate buffer. For rosettasome binding experiments, a 25 mM Hepes buffer (pH 7.5) containing 200 mM K_2SO_4 was used. For proton leakage experiments liposomes made from *Sulfolobus* lipids or POPC/POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine with 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] at a ratio of 20:1, by weight) were prepared in 100 mM Hepes buffer with 250 mM K_2SO_4 and 1 mM 8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine) at pH 6.5. Liposomes were made by extruding lipid suspensions 13 times through two polycarbonate membranes (pore size = 0.1 μm) at 70°C for *Sulfolobus* lipids and 25°C for POPC/POPG. For proton leakage experiments, the nonencapsulated fluorophore was removed by using a Sephadex G25 column equilibrated in buffer without pyranine.

In liposome binding experiments, purified rosettasomes were mixed with liposomes in 25 mM Hepes buffer (pH 7.5) containing 200 mM K_2SO_4, 1 mM ATP, and 25 mM $MgCl_2$. The mixture (25 μl) was incubated at room temperature or at 60°C for 10 min before centrifuging at 60,000 rpm for 15 min at 20°C in a TLA 100 rotor (Beckman). After centrifugation, the supernatant was removed immediately and the liposome-containing pellet was resuspended in a volume of buffer equal to the volume of the supernatant. Proteins in the supernatant and pellet were analyzed by Alton PAGE as described (20).

The effects of rosettasome binding on lipid phase transitions (T_m) were measured by using a Microcal (Northhampton, MA) VP-DSC. All samples were degassed under dynamic vacuum at 25°C by using a Microcal ThermoVac before scanning. Liposomes made from DPPC/G (dipalmitoyl-phosphatidylcholine with dipalmitoyl-phosphatidylglycerol, 20:1 ratio by weight) were prepared at a concentration of 0.25 mg/ml in 25 mM Hepes, pH 7.5 as described above. Samples of liposomes alone, liposomes plus 1 mM ATP, liposomes plus 25 mM $MgCl_2$, liposomes plus 1 mM ATP and 25 mM $MgCl_2$, liposomes plus recombinant rosettasome subunits (α/β 1:1 mixture, 1 mg/ml), liposomes plus 1 mM ATP, 25 mM $MgCl_2$ and recombinant rosettasomes (α/β 1:1 mixture, 1 mg/ml), and liposomes plus purified rosettasomes at 1 mg/ml, were scanned at 1°C per min from 20°C to 65°C.

Proton leakage experiments were done basically as described (26). Briefly, 15 μl of the liposome suspension was placed in a cuvette with 1.2 ml of 100 mM Hepes, 250 mM K_2SO_4. In various trials, this buffer also contained some combination of 10 μM valinomycin, 1 mg/ml rosettasome proteins, 1 mM ATP, and 25 mM $MgCl_2$. The solution was equilibrated to the experimental temperature for 5 min with stirring and then raised to pH 7.5 by the addition of a calibrated volume (12.5 μl) of ≈4 M NaOH. The efflux of protons from the liposomes was monitored by fluorescence emission of pyranine at 511 nm by using a Fluoromax 2 fluorometer (Jobin-Yvon, Horiba, NJ).

Results

Rosettasomes Localization *in Vivo*. Assuming that rosettasomes are functionally related to other chaperonins and involved in protein folding as reported (11), we anticipated they would be located in the cytoplasm, where most protein folding is presumed to occur. To our surprise, however, using polyclonal antibodies in both IFM and immunogold electron microscopy (IEM), we observed rosettasomes predominantly distributed around the inside of the cell membrane (Fig. 1). By IFM rosettasome fluorescence created a distinct glow around the membrane in the vast majority of cells (Fig. 1*A*). Similarly, by IEM the rosettasome-bound gold particles accumulated around the perimeter of cells (Fig. 1*B*). Both of these results are characteristic of membrane-associated proteins (54).

By IFM, we examined cultures grown at 60°C and 76°C, and

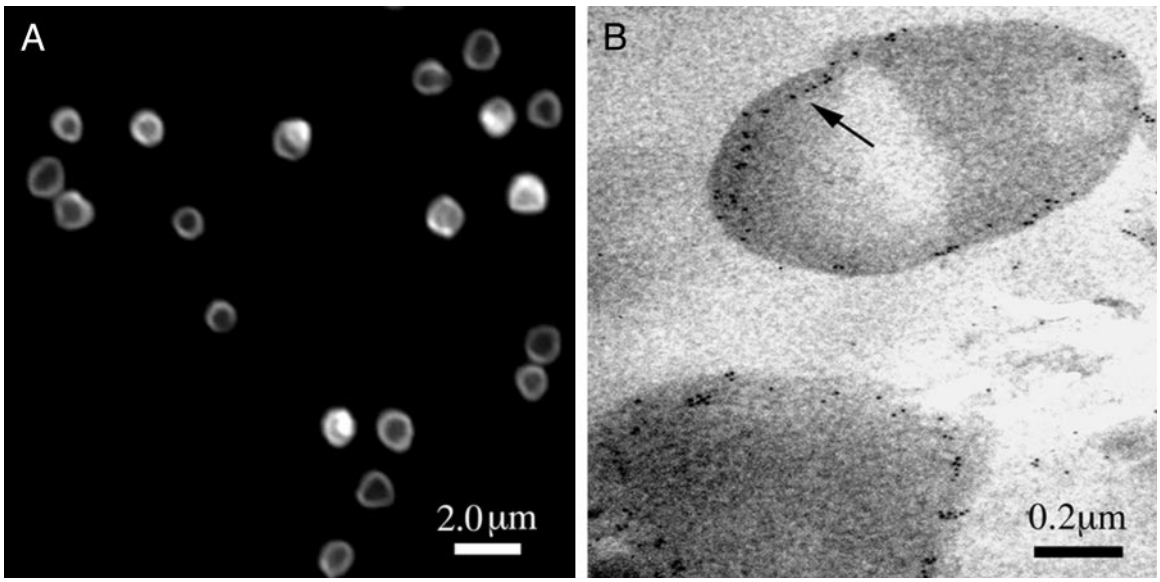

Fig. 1. Immunological localization of HSP60s in *S. shibatae* using fluorescence microscopy and transmission electron microscopy. (*A*) In general, the fluorescence signal outlines the perimeter of cells, which is indicative of membrane localization. (*B*) In sections of Epon-embedded cells, the 10-nm ImmunoGold particles (arrow) also outlined the perimeter of cells.

76°C heat-shocked at 85°C for 3.5 h to determine whether rosettasome composition, which is known to be temperature dependent (20), influenced its localization. In all cases the distribution of rosettasomes remained qualitatively the same as shown in Fig. 1*A*, although fluorescence intensity increased in heat-shocked cells. This increase after heat shock is expected from the increased abundance of rosettasome proteins that occurs during heat shock (see refs. 10 and 20). By immunogold electron microscopy, we examined cultures grown at 76°C frozen within milliseconds by high-pressure techniques, freeze substituted and embedded. We made thick sections (0.25–1.0 μm) that were etched to enhance antibody reactions (22), and rosettasomes were located by using 10-nm immunogold by transmission electron microscopy (Fig. 1*B*). In all cells the majority of gold particles marking the position of rosettasomes were distributed around the perimeter of the cells. Like the IFM result, this is indicative of an association between rosettasomes and the inside of the cell membrane.

Heat Shock Decreases Membrane Leakage at Lethal Temperature. We had previously reported that heat-shocked *S. shibatae*, like other organisms, show improved survival at lethal temperatures and that the proline analogue, azetidine-2-carboxylic acid, which prevents functional protein synthesis, interferes with this enhanced survival (9). In light of our observations that rosettasomes are distributed around the cell membrane, we investigated if there are temperature-dependent changes in membrane permeability in *S. shibatae* (Fig. 2). Using propidium iodide as an indicator of membrane porosity, we observed that indeed heat-shocked cells (exposed to 85°C for 1 or 2 h) were significantly less permeable than normal (76°C-grown) cells, when exposed to a lethal temperature (92°C) (Fig. 2*A*). In normal cultures shifted to lethal temperature, >50% of the cells were propidium iodide permeable in 2 h and >90% in 3–4 h, whereas in heat-shocked cultures <25% of the cells were permeable in 2 h and <40% in 4 h.

The enhanced integrity of the membrane by heat shock was nearly eliminated in the presence of the proline analogue azetidine-2-carboxylic acid, indicating that functional protein synthesis is needed unless azetidine itself caused membrane leakage (Fig. 2*B*). Adding azetidine at the end of the heat shock period, however, gave similar results to heat shock (Fig. 2*C*), indicating that the azetidine itself did not cause membrane leakage. Published findings that the rosettasome proteins are nearly the only proteins synthesized during heat shock (10) suggest that azetidine is primarily influencing functional rosettasomes, unless the membrane itself is changing.

Temperature-Dependent Changes in Membrane Composition. It is known that *Sulfolobus* spp., like other organisms, change the composition of their membranes in response to environmental temperatures (27). Unlike bacteria, however, which adjust their membrane fluidity by changing the saturation state of their ester-linked fatty acyl chains, *Sulfolobus* spp. and other archaea adjust fluidity by inserting cyclopentane groups in their tetraether linked C40 dibiphytanyl chains (for review see ref. 28). To determine whether such membrane changes could explain the changes we observed in permeability after heat shock, we investigated membrane composition at normal, heat shock, and lethal temperatures and during heat shock in the presence of azetidine (Fig. 3). We observed that with increasing durations of heat shock (85°C for 1–6 h), the percentages of C40 chains with one cyclopentane ring (1R) decreased, whereas those with two and three rings (2R and 3R) increased (Fig. 3*A*), indicating that membrane changes were occurring during heat shock. Exposing 76°C-grown cells directly to lethal temperatures, however, caused nearly identical changes in lipid composition to the changes observed during heat shock (Fig. 3*B*). Hence the changes in membrane permeability we observed in propidium iodide experiments were not caused by changes in membrane composition. Membrane composition appears to be the same in cells shifted from 76°C to 92°C or heat-shocked, but propidium iodide experiments indicated that membrane leakage was different (compare Fig. 2*A*).

We considered the possibility that rosettasomes interact with lipid-modifying enzymes, increasing their stability and efficiency during heat shock. If this is true, then azetidine, which we observed impacted membrane permeability (Fig. 2*B*), would also impact membrane composition. We compared the membrane composition of cells heat shocked with and without azetidine and observed no significant difference in lipid composition (Fig. 3*C*). This finding indicated that lipid-modifying enzymes were not effected by azetidine nor did they depend on

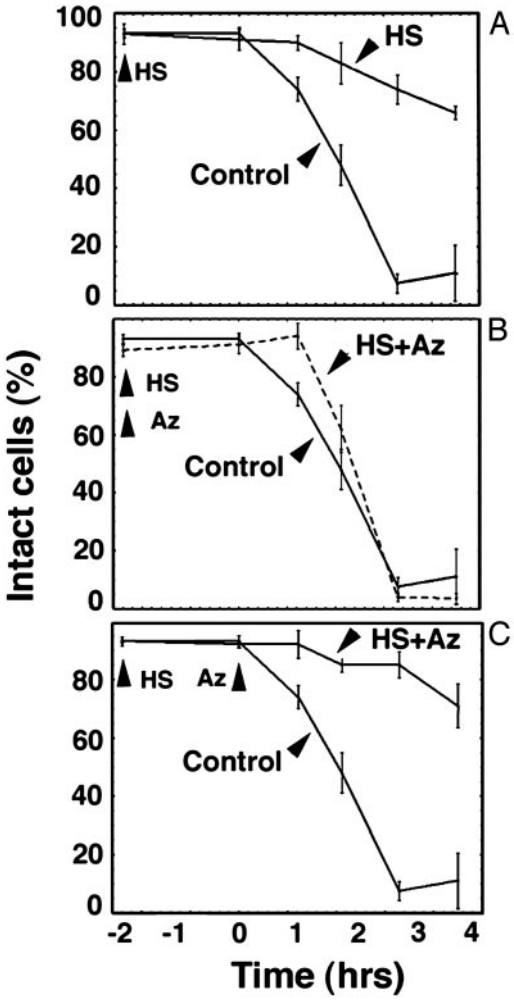

Fig. 2. The membrane integrity of *S. shibatae* cells with increasing exposures to lethal temperature (92°C) indicated by the percentage of cells able to exclude propidium iodide. (*A*) Cultures shifted to lethal temperature at time 0 either directly from 76°C (control) or after 2 h of heat shock (HS) at 86°C. (*B*) Heat shock (HS) in the presence of the amino acid analogue, azetidine (Az) showed minimal improvements over controls. (*C*) The addition of azetidine (Az) after heat shock indicated that azetidine itself does not cause membrane leakage. The error bars show the standard deviation of the mean ($n = 3$).

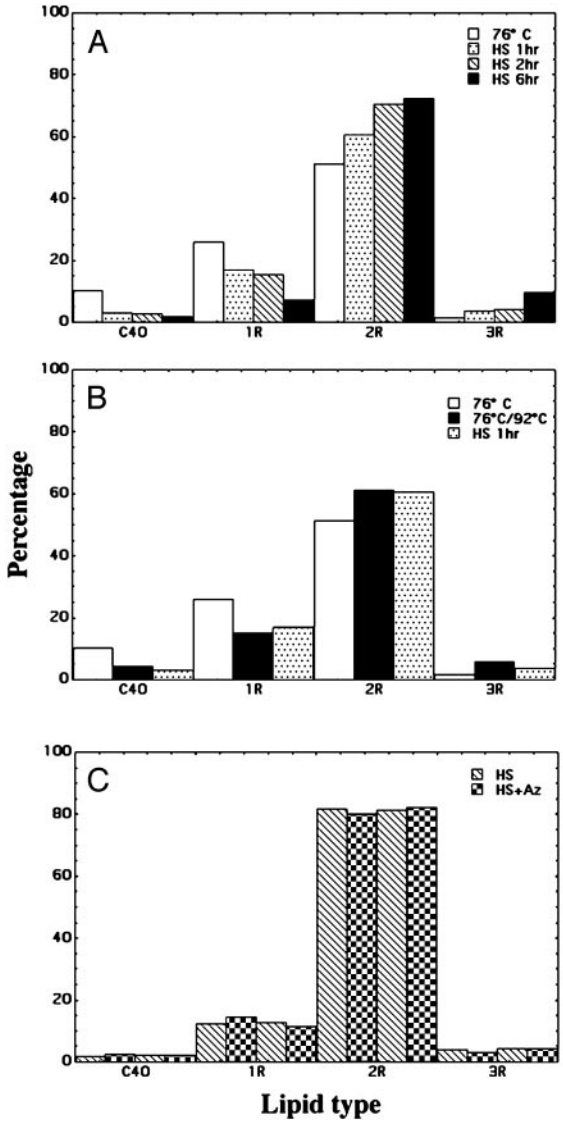

Fig. 3. Temperature-dependent changes in the composition of *S. shibatae* membranes. (*A*) The membranes of cultures grown at 76°C and heat-shocked at 85°C for 1, 2, or 6 h change in their percentage of C_{40} biphytane, and C_{40} with one, two, or three cyclopentane groups (1R, 2R, 3R, respectively). (*B*) The percentage of C_{40} chains with 0, 1R, 2R, and 3R in 76°C cultures compared to cultures exposed directly to lethal temperature (92°C) for 1 h or heat shocked (85°C) for 1 h. (*C*) Replicate experiments indicate that the changes in C_{40} composition of membranes during heat shock were not influenced by the presence of azetidine-2-carboxylic acid.

functional rosettasomes, supporting the conclusion that membrane composition alone does not explain the observed temperature-dependent changes in membrane permeability.

Rosettasomes Bind to Liposomes. Using rosettasomes with different subunit compositions, purified from *S. shibatae* grown at different temperatures (20) and liposomes made from *Sulfolobus* lipids (29), we observed that rosettasomes bind to liposomes (see Fig. 5, which is published as supporting information on the PNAS web site). Similar results were obtained with liposomes made from a variety of other lipids, including diether lipids from halophilic archaea, POGP (palmitoyl-oleoyl-glycero-phosphocholine) and DOPG (dioleoyl-glycero-phospho-RAC-glycerol), DOPC (dioleoyl-glycero-phosphotidycholine), DPPC/G (dipalmitoyl-phosphatidylcholine with dipalmitoyl-phosphatidylglycerol), DMPC (dimystri-phosphatidylcholine), and the negative and positive lipid mixtures in liposome kits (Sigma). In all liposome experiments, the α, β, and γ subunits did not bind to liposomes unless they were assembled into double rings or filaments. This meant that pure α or pure β or mixtures of α and β or α, β, and γ bind, but pure γ, which does not form double rings (20), did not bind. This interaction between rosettasomes and lipids expands the possibilities for how these chaperonins interact with biological membranes but does not prove that they do so through the lipids.

To determine whether rosettasomes influence the ordering of lipids, we experimented with liposomes made from lipids with sharp endothermic transitions (T_m). Using model lipids, such as a mixture of dipalmitoyl-phosphatidylcholine and dipalmitoyl-phosphatidylcholine, we measured phase transitions with and without bound rosettasomes by differential scanning calorimetry (DSC). In these liposome systems, we observed no measurable effects of rosettasome binding, suggesting that the presence of the rosettasome does not impact the physical ordering of the acyl chains within the hydrophobic interior of the lipid. DSC also

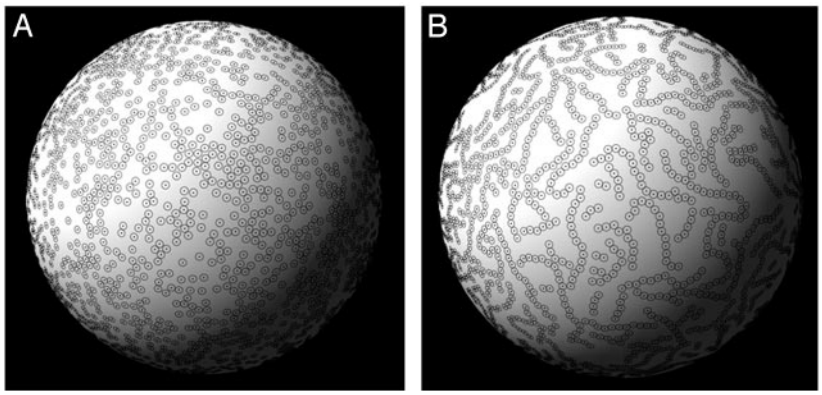

Fig. 4. The distribution of the estimated 4,600 rosettasomes on spheres representing *S. shibatae* cells (drawn to scale by using 17.2-nm rosettasome rings and 1-μm *S. shibatae* cells). (*A*) Rosettasomes are represented by randomly distributed, nonoverlapping disks that cover ≈34% of the surface. (*B*) To represent rosettasome filaments, disks were fused at two diametrically opposed points (arbitrarily set to 1 nm) by simulating random motion and arbitrarily attaching them. Filaments will cover ≈39% of the surface.

indicated that rosettasome binding had no detectable effects on the enthalpy (ΔH) for the phase transitions of these liposomes.

We reasoned that *S. shibatae,* living at pH 2 and maintaining an intracelluar pH of 6.5 (27), may produce large amounts of membrane-associated rosettasomes to help regulate proton fluxes. Using liposomes made from both model lipids and *Sulfolobus* lipids, we investigated whether rosettasomes could measurably influence proton leakage by a fluorescence method (30). In experiments with POPC/POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine with 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] liposomes at temperatures of 20°C and 60°C and *Sulfolobus* liposomes at 25°C and 60°C, we observed no significant effect of rosettasomes on rates of proton leakage.

Discussion

In response to high-temperature stress most organisms, including most archaea, increase the synthesis of a variety of HSPs (31, 32). In contrast, *S. shibatae* increases the synthesis of almost exclusively HSP60s, which are among its most abundant proteins under all conditions (10). This makes *S. shibatae* an ideal organism for studying HSP60 function *in vivo*. In studies reported here, the HSP60s in *S. shibatae,* which form rosettasomes, are shown to be membrane associated. There are previous reports that HSP60s in bacteria and mitochondria, which form group I chaperonins, are membrane associated (33–37). There are also reports that the HSP60-related TCP1s in eukarya, which form group II chaperonins (known as CCT or TriC), are membrane associated (38–41). We recently discovered that in human red blood cells TCP1 is predominantly cytoplasmic under normal conditions, but after heat treatment it relocates to the membrane (C. T. Wagner, I. Y. Lu, M. H. Hoffman, W. Q. Sun, J.D.T., and J. Connor, unpublished work). We found no previous reports that HSP60s in archaea, which are also classified as group II chaperonins, are membrane associated.

Although it is generally believed that the function of all chaperonins is to fold or refold proteins (for a recent review see ref. 13), the association of at least some chaperonins with the cell membrane suggests to us they have membrane-related functions. Such functions may include a role in folding membrane-associated proteins, as has been suggested (35, 42). There is also evidence for other membrane-related functions, such as binding and stabilizing lipids (43) and intracellular and intercellular signaling (44, 45). In light of observations reported here and previously (46), we hypothesize that the rosettasome chaperonins have a structural function, acting as a kind of membrane skeleton.

Based on observations of cell morphology in solution and morphological changes on surfaces, Searcy and Hixon (47) suggested that *Sulfolobus* spp. must have some kind of cytoskeleton. Baumeister and Lembcke (48) proposed that the *Sulfolobus* surface layer proteins attached to an intracellular network could account for Searcy and Hixon's observations and stabilize the cell membrane. To determine whether rosettasomes could form such a membrane network, we calculated their potential surface distribution by using the formula: percent sphere coverage = (100) × (no. of rings) × (ring area)/(sphere surface area) (Fig. 4). Using published estimates of rosettasome abundance (4,600 per cell) and size (17.2 nm) (46) and calculating surface area based on a *S. shibatae* diameter of 1.0 μm, the rings cover ≈34% of the sphere's surface, assuming all rosettasomes are membrane associated and randomly distributed without overlapping (Fig. 4*A*). We refined this calculation in light of evidence that rosettasomes form filaments *in vivo* (46) by allowing the rings to move randomly and interact through two diametrically opposed "sticky regions" (Fig. 4*B*). Rosettasome filaments, which form through apical domain interactions, are somewhat larger than rings and cover ≈39% of the sphere's surface.

Both of these simulations were done with estimates of rosettasome abundance under normal conditions. After heat shock, however, densitometry analyses indicate that rosettasome abundance increases ≈2.5 times (H.K.K. and J.D.T., unpublished work). Thus under normal conditions rosettasomes may cover more than a third of the cell surface and after heat shock this may increase to >50% coverage.

The reported composition of archaeal membranes is 60% protein, 25% lipid, and 10% carbohydrate (49). Considering their potential coverage, rosettasome rings or filaments may be expected to interact with membrane proteins and/or a major part of the lipid surface. Indeed there is evidence that rosettasomes interact with proteins (11, 15), although membrane proteins have yet to be specifically investigated. Our liposome experiments establish that rosettasomes interact with lipids, but the nature and consequences of this interaction require further study. We observed no effect on lipid transition temperatures, although Tsvetkova *et al.* (50) have shown that small HSPs bind lipids and effect phase transitions. We found no measurable influence of rosettasome binding on proton permeability of liposomes. It is known, however, that high temperatures influence membrane permeability (51–53), suggesting that further investigations using more complex membranes are needed to model whether rosettasomes impact proton fluxes.

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